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A QUANTITATIVE STUDY OF HEMOLYTIC SERUM.*

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ONE of the most important problems of experimental medicine today is the determination of the rôle of physico-chemical laws in the chemistry of disease. The controversy over this question between Ehrlich on the one side and Arrhenius and Madsen on the other, touches many problems of practical medicine. Unfortunately, most of the toxic and antitoxic substances with which these investigators are working, are substances not yet isolated in a state of purity, are of unknown composition and probably of great complexity. Hence their study offers many difficulties.

It was thought that if a "border line" phenomenon were studied, some problem lying partly in the well-trodden field of inorganic chemistry, where the application of physico-chemical laws cannot be gainsaid, light might be thrown on the more complex phenomena now engaging the attention of these workers. For this reason, and at the suggestion of Dr. Hektoen, the study of the neutralization of a hemolytic serum with certain salt solutions was undertaken.

It was found comparatively easy to modify the technique of the hemolytic experiment so as to give to it an accuracy equal to that of the most exact hemoglobin determination, and by this means to plot neutralization curves for such sera. These curves, however, showed unlooked-for complexities.

In order to interpret them it was found necessary to plot corresponding curves showing the changes in hemolytic power as each of the prominent serum components varies. These preliminary curves are of such interest that it is thought best to embody them in a separate report. The present paper deals only with these curves.

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NOMENCLATURE.

So much confusion has resulted from failure to separate demonstrated facts from hypotheses and theories in immunity, that it is thought best to define certain terms used in this paper. The term **amboceptor** is used to designate the specific immune body present in an immune serum. This is the "substance sensibilatrice" of Bordet, the intermediary body of Ehrlich, the "copula" of Müller. It is a thermostable substance, resisting heat to at least 57° C. for over an hour. By itself it has no bactericidal or hemolytic action. It produces, however, certain physical or chemical changes in bacteria and other cells, which render them susceptible to the action of a second serum component, the complement. Cells thus rendered susceptible are spoken of as being **sensitized.**

The **complement** is a normal component of serum. It is the "alexin" of Bordet, the "cytase" of Metchnikoff. It is a thermolabile substance, usually destroyed by heat to 55°C. for 30 minutes. By itself it has no bactericidal or hemolytic action. It is capable of producing such action only in the presence of amboceptor or when acting on cells previously sensitized by amboceptor.

These terms are selected because they are the ones most commonly used in American medical literature. They are used here simply as names for unknown chemical substances, with no implication as to the method of action or interaction of these substances.

MATERIAL AND TECHNIQUE.

The serum used in this study was obtained by immunizing goats by repeated intraperitoneal injections of washed sheep corpuscles. This serum was selected because Hektoen and Ruediger found that normal goat serum is without hemolytic action on sheep corpuscles, and because Ehrlich and Morgenroth[1] report that after immunization such serum has little or no agglutinating action, an action that interferes seriously with exact hemolytic work.*

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[1] *Berl. klin. Wchnschr.*, 1899, 36, p. 481.

*Of the five normal goats used, four showed no hemolytic action on sheep corpuscles. The fifth gave a slight trace of hemolysis when its serum was used in large amounts. Of the two immune goats, one gave practically no agglutination of these corpuscles. The second gave at first a moderate amount of agglutination, which amount became less and nearly disappeared as the serum increased in hemolytic power.

Blood was drawn from the jugular vein of these animals by means of a sterile hypodermic needle through sterile rubber tubing, into a sterile Erlenmeyer flask, a suction-pump being used to hasten the flow. The sheep's blood was at once defibrinated by gently stirring it with a sterile glass rod or by shaking it with sterile glass beads. The goat's blood was allowed to clot, and was kept in a refrigerator till the serum separated. Serum free from complement was prepared by heating this serum to 57°C. for one hour.

The salt solution used in the work was made from water redistilled from glass, and extra pure NaCl obtained from the Department of Chemistry. The solution was eighth-molecular in strength and was sterilized in the autoclave* before being used.

In performing the hemolytic experiment, the following technique was finally adopted and was used in all the work herein reported. Test-tubes of a uniform internal diameter of 1.3 cms. were selected, were carefully cleaned, plugged with absorbent cotton made non-absorbent by treatment with a weak ethereal solution of paraffin, and sterilized by dry heat (150° C. for one hour). With strictest aseptic precautions the sera and other solutions to be used were measured out into these test-tubes, and the volumes in the various tubes made up to a uniform amount, five c.c. The tubes were repeatedly shaken to insure a thorough mixing of the solutions.

After standing for an hour at room temperature, 3.5 c.c. of a seven per cent suspension of washed sheep corpuscles† were added to each tube and the tube immediately inverted once or twice to bring about a prompt, thorough and uniform mixing of the corpuscles and serum, the paraffin preventing adhesion of the fluid to the cotton plugs. The tubes were then incubated at 37.5° C. for three hours, after which they were placed in the refrigerator till the corpuscles settled. This generally took place in about 36 hours.

To estimate the amount of hemolysis a color-scale was prepared by laking corpuscles in distilled water in such amounts that 100 per cent on the scale represented a complete solution of all corpuscles entering into a hemolytic tube. The fluids of this color-scale were placed in test-tubes the same diameter as the hemolytic tubes, and comparisons made in a darkened room by holding tubes side by side against a strongly illuminated piece of white paper as a background.

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*Contamination by condensation water prevented by small beaker inverted over neck of flask.

†Corpuscles were washed four times in from five to six times their volume of salt solution. Supernatant liquid removed each time with a sterile capillary tube and suction pump. Calculated residual serum less than 0.00001 c.c. per hemolytic tube.

Before estimating the amount of hemolysis the liquid in each tube was mixed to insure a uniformity of color throughout. To do this the tube was gently tipped so as to allow a bubble of air to rise to the bottom. By careful manipulation, a thorough mixing can be brought about by this means, without disturbing the corpuscles at the bottom of the tube. After the first reading, the tubes were replaced in the refrigerator and the tints estimated again, 24 hours later. The average of the 36- and 60-hour readings was taken as the **observed hemolysis.**

To obtain the **true** or **calculated hemolysis** there was deducted from the observed hemolysis a percentage representing the sum of the tint of the serum originally added to the tube and the amount of autolysis taking place in control tubes. This sum was rarely over two per cent.

In the earlier experiments, considerable trouble was experienced on account of an excess of this autolytic action.* Part of this, it was thought, might possibly be due to faulty cleaning of glassware. To remedy this, every piece of glassware entering into the experiments was subjected to the following routine treatment. Before being used the first time, it was boiled for an hour in a strong solution of NaOH and soap, was scrubbed free from visible dirt, rinsed repeatedly in hot water and soaked for 24 hours in 10 per cent HCl. It was then rinsed in distilled water, soaked in distilled water for 24 hours, dried, plugged with chemically pure, non-absorbent cotton, and sterilized in the hot air sterilizer. During subsequent cleanings the same routine was used, except that the preliminary boiling in NaOH was usually omitted.

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*The corpuscles of different sheep differ in the amount of this autolysis. The autolysis was so excessive with two sheep that the use of their corpuscles had to be discontinued. The autolysis apparently varies in the same animal with change in exercise and diet. The autolysis is completely inhibited by adding either of the antilytic salts $CaCl_2$ or $BaCl_2$. Other salts were not tried. One of the discarded sheep gave practically no autolysis when 0.85 per cent NaCl-solution was used. This was not true with the second discarded sheep. With all sheep, the spontaneous laking (autolysis) in $\frac{m}{8}$ NaCl decreased with repeated bleedings, and eventually practically disappeared.

A short study of autolysis showed that the larger part of it took place during the first 24 hours. Thus, with one sheep, during the first 24 hours' suspension in $\frac{m}{8}$ NaCl, there was a spontaneous laking of 1.5 per cent, during the second 24 hours a laking of 0.7 per cent, and during the third but 0.5 per cent. In order to reduce the autolytic tint to a minimum, the corpuscles were, therefore, freed from serum the afternoon of their withdrawal and allowed to stand in salt solution over night, when the major part of the autolysis took place. They were washed free from the tinted fluid immediately before being used the next forenoon. By this means control tubes were obtained, the combined autolytic and serum tint of which was often less than one per cent.

THE COMPLEMENT.

The first experiment to determine the changes in hemolytic power as the complement varies, was done with sensitized corpuscles. These were prepared by exposing washed corpuscles to heated immune serum, for an hour, at room temperature. The corpuscles were then freed from serum by centrifugation and washed repeatedly in salt solution. Increasing quantities of 20 per cent normal serum (complement) were added to a series of test-tubes and the volumes made up to a constant quantity (five c.c.) with 20 per cent heated normal serum (no complement). This gave a series of tubes each containing the same amounts of inorganic salts, serum albumin, etc., but differing in their content of the thermolabile substances, among which is the complement. 3.5 c.c. of a seven per cent suspension of sensitized corpuscles were then added to each tube, the tubes incubated, placed in the refrigerator, and tints estimated as described above. The result is recorded in Table 1 and shown graphically in Fig. 1.

TABLE 1.
DATE FOR COMPLEMENT CURVE. (See Fig. 1.)

	COMPLEMENT (measured in c.c.'s of normal serum)											
	0.0	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0 c.c.
36-hr. reading.	4.0	6.0	7.0	15.0	35.0	65.0	76.0	85.0	85.0	95.0	98.0	100.0
60-hr. reading.	4.0	6.0	8.0	17.5	45.0	70.0	80.0	85.0	88.0	95.0	100.0	100.0
Average	4.0	6.0	7.5	16.25	40.0	67.5	78.0	85.0	86.5	95.0	99.0	100.0
Autolysis + serum tint..	4.0						4.0					4.0
Calculated....	**0.**	**2.0**	**3.5**	**12.25**	**36.0**	**63.5**	**74.0**	**81.0**	**82.5**	**91.0**	**95.0**	**(100)**

TABLE 2.

DATA FOR COMPLEMENT CURVES. (See Fig. 6.)

		COMPLEMENT (Measured in c.c.'s of 66⅔ Per Cent Normal Serum)																				
		0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.125	1.25	1.375	1.5	1.75	2.0	2.25	2.5	2.75	3.0
Serum tint		**1.5**	....	....	....	....	....	....	....	....	....	**1.75**	....	....	....	....	....	**2.0**	....	....	....	**2.25**
0.00 c.c. Amboceptor (control)	36-hr. reading	1.5	....	....	....	....	1.5	....	....	....	....	1.5	....	....	....	1.5	....	2.0	....	2.5	....	2.5
	60-hr. reading	2.5	....	....	....	....	2.5	....	....	....	....	2.5	....	....	....	2.5	....	3.0	....	3.5	....	4.5
	Average	**2.0**	....	....	....	....	**2.0**	....	....	....	....	**2.0**	....	....	....	**2.0**	....	**2.5**	....	**3.00**	....	**3.5**
0.01 c.c. Amboceptor	36-hr. reading	2.0	2.5	2.5	3.0	3.0	4.0	4.0	5.0	6.0	7.0	7.5	9.0	12.5	14.0	13.0	15.0	16.0	17.5	19.0	22.5	25.0
	60-hr. reading	2.5	3.0	3.5	5.0	5.0	5.0	6.0	7.0	7.5	8.0	10.0	11.0	13.0	14.0	15.0	16.0	18.0	20.0	22.5	25.0	27.5
	Average	2.25	2.75	3.0	4.0	4.0	4.5	5.0	6.0	6.75	7.5	8.75	10.0	12.75	14.0	14.0	15.5	17.0	18.75	20.75	23.75	26.25
	Calculated	**0.25**	**0.75**	**1.0**	**2.0**	**2.0**	**2.5**	**3.0**	**4.0**	**4.75**	**5.5**	**6.75**	**8.0**	**10.75**	**12.0**	**12.0**	**13.25**	**14.5**	**16.0**	**17.75**	**20.5**	**22.75**
0.02 c.c. Amboceptor	36-hr. reading	2.0	2.0	3.0	4.0	7.5	9.0	15.0	17.5	27.5	35.0	37.5	42.5	45.0	47.5	52.5	59.0	65.0	67.5	72.0	75.0	82.0
	60-hr. reading	2.5	3.0	4.0	6.0	7.0	10.0	15.0	17.5	27.5	37.5	42.5	47.5	52.5	54.0	56.0	57.5	60.0	65.0	70.0	75.0	80.0
	Average	2.25	2.5	3.5	5.0	7.25	9.5	15.0	17.5	27.5	36.25	40.0	45.0	48.75	50.75	54.25	58.25	62.5	66.25	71.0	75.0	81.0
	Calculated	**0.25**	**0.5**	**1.5**	**3.0**	**5.25**	**7.5**	**13.0**	**15.5**	**25.5**	**34.25**	**38.0**	**43.0**	**46.75**	**48.75**	**52.25**	**56.0**	**60.0**	**63.5**	**68.0**	**71.75**	**77.5**
0.03 c.c. Amboceptor	36-hr. reading	2.0	2.5	3.0	5.0	8.0	15.0	25.0	32.5	47.5	60.0	65.0	68.0	72.0	75.0	77.5	80.0	82.5	85.0	88.0	93.0	98.0
	60-hr. reading	2.5	3.0	5.0	6.0	9.0	16.0	26.0	35.0	47.5	57.5	65.0	70.0	72.0	73.0	75.0	77.5	80.0	82.5	90.0	95.0	100.0
	Average	2.25	2.75	4.0	5.5	8.5	15.5	25.5	33.75	47.5	58.75	65.0	69.0	72.0	74.0	76.25	78.75	81.25	83.75	89.0	94.0	99.0
	Calculated	**0.25**	**0.75**	**2.0**	**3.5**	**6.5**	**13.5**	**23.5**	**31.75**	**45.5**	**56.75**	**63.0**	**67.0**	**70.0**	**72.0**	**74.25**	**76.5**	**78.75**	**81.0**	**86.0**	**91.75**	**95.5**
0.04 c.c. Amboceptor	36-hr. reading	2.0	3.0	3.5	7.5	15.0	27.5	42.5	55.0	62.5	68.0	70.0	75.0	80.0	80.0	82.5	82.5	87.5	92.5	95.0	100.0	100.0
	60-hr. reading	2.5	4.0	5.5	8.0	12.5	26.0	45.0	57.5	65.0	70.0	74.0	79.0	85.0	85.0	85.0	87.5	90.0	95.0	97.5	100.0	100.0
	Average	2.25	3.5	4.5	7.75	13.75	26.75	43.75	56.25	63.75	69.0	72.0	77.0	82.5	82.5	83.75	85.0	88.75	93.75	96.25	100.0	100.0
	Calculated	**0.25**	**1.5**	**2.5**	**5.75**	**11.75**	**24.75**	**41.75**	**54.25**	**61.75**	**67.0**	**70.0**	**75.0**	**80.5**	**80.5**	**81.75**	**82.75**	**86.25**	**91.0**	**96.25**	**96.75**	(100)
0.05 c.c. Amboceptor	36-hr. reading	2.5	3.0	5.0	12.0	21.0	45.0	60.0	64.0	70.0	75.0	79.0	80.0	82.5	86.0	86.0	89.0	95.0	100.0	100.0	....	....
	60-hr. reading	2.5	5.0	6.0	11.0	22.5	42.5	65.0	70.0	75.0	77.5	81.0	85.0	87.5	94.0	95.0	97.5	100.0	100.0	100.0	....	....
	Average	2.5	4.0	5.5	11.5	21.75	43.75	62.25	67.0	72.25	76.25	80.0	82.5	85.0	90.0	90.5	93.25	97.5	100.0	100.0	....	....
	Calculated	**0.5**	**2.0**	**3.5**	**9.5**	**19.75**	**41.75**	**60.25**	**65.0**	**70.25**	**74.25**	**78.0**	**80.5**	**83.0**	**88.0**	**88.5**	**91.0**	**95.0**	**97.25**	**(100)**	....	....
0.06 c.c. Amboceptor	36-hr. reading	2.0	3.0	6.0	15.0	27.5	60.0	75.0	80.0	85.0	90.0	90.0	91.0	96.0	95.0	95.0	100	100	....	....	....	....
	60-hr. reading	3.0	6.0	6.5	14.0	27.5	60.0	75.0	82.5	85.0	87.5	90.0	93.0	95.0	95.0	97.0	99	100	....	....	....	....
	Average	2.5	4.5	6.25	14.5	27.5	60.0	75.0	81.25	85.0	88.75	90.0	92.0	95.5	95.0	96.0	99.5	100	....	....	....	....
	Calculated	**0.5**	**2.5**	**4.25**	**12.5**	**25.5**	**58.0**	**73.0**	**79.25**	**83.0**	**86.75**	**88.0**	**90.0**	**93.5**	**93.0**	**94.0**	**97.25**	**(100)**	....	....	....	....
0.08 c.c. Amboceptor	36-hr. reading	2.0	3.0	10.0	25.0	58.0	85.0	90.0	92.5	95.0	100.0	100.0	....	....	....	....	....	....	....	....	....	....
	60-hr. reading	3.0	6.0	10.0	26.0	60.0	85.0	87.5	95.0	95.0	97.5	100.0	....	....	....	....	....	....	....	....	....	....
	Average	2.5	4.5	10.0	25.5	59.0	85.0	88.75	93.75	95.0	98.75	100.0	....	....	....	....	....	....	....	....	....	....
	Calculated	**0.5**	**2.5**	**8.0**	**23.5**	**57.0**	**83.0**	**86.75**	**91.75**	**93.0**	**96.75**	**98**	....	....	....	....	....	....	....	....	....	....
0.12 c.c. Amboceptor	36-hr. reading	2.5	6.0	17.5	52.0	85.0	100.0	100.0	100.0	....	....	....	....	....	....	....	....	....	....	....	....	....
	60-hr. reading	3.0	4.0	17.5	50.0	85.0	100.0	100.0	100.0	....	....	....	....	....	....	....	....	....	....	....	....	....
	Average	2.75	5.0	17.5	51.0	85.0	100.0	100.0	100.0	....	....	....	....	....	....	....	....	....	....	....	....	....
	Calculated	**0.75**	**3.0**	**15.5**	**49.0**	**83.0**	(100)	**100+**	**100+**	....	....	....	....	....	....	....	....	....	....	....	....	....

A similar experiment with non-sensitized corpuscles gave the curve in Fig. 2. In this experiment increasing quantities of $33\frac{1}{3}$ per cent normal serum were added to a series of test tubes, the volumes made up to a constant amount (three c.c.) with a $33\frac{1}{3}$ per cent heated normal serum, and the same amount of amboceptor (two c.c. 30 per cent heated immune serum No. 1) added to each. After thoroughly mixing and allowing to stand for an hour at room temperature, 3.5 c.c. of a seven per cent suspension of corpuscles were added to each tube and the tubes treated as above. The curve obtained in this way differs so little from that with sensitized corpuscles, that the use of sensitized corpuscles was discontinued in this work.

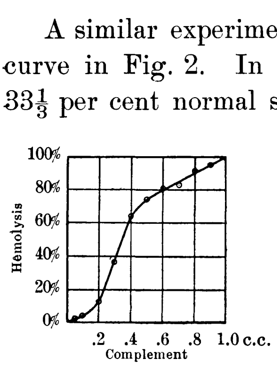
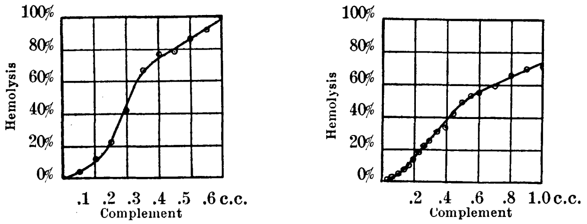


FIG. 1.—Complement curve with sensitized corpuscles.

A third curve, obtained in the same way but with a smaller constant amount of amboceptor (two c.c. 15 per cent heated immune serum No. 1) is shown in Fig. 3.



FIGS. 2 AND 3.—Complement curves with constant amboceptor.

To determine the influence of the amount of amboceptor on the nature of these curves, parallel curves were run, with the same varying amounts of complement but with different constant amounts of amboceptor. Two curves obtained in this way are shown in Fig. 4. In the upper curve 0.05 c.c. of heated immune serum (No. 2) was used as amboceptor; in the lower curve, but half that amount. A similar set of curves is shown in Fig. 5. The upper one was made with 0.15 c.c. of heated immune serum (No. 1), the lower with 0.075 c.c. of the same serum.

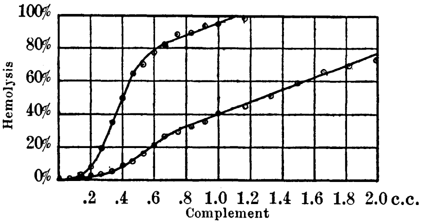


FIG. 4.—Parallel complement curves. (Immune Serum No. 2, Normal Serum No. 4.)

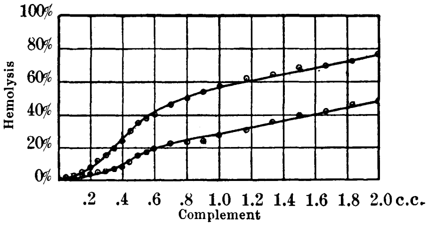


FIG. 5.—Parallel complement curves. (Immune Serum No. 1, Normal Serum No. 3.)

Two more extensive series of curves are shown in Figs. 6 and 7. The readings and calculations for the curves in Fig. 6 are given in Table 2.

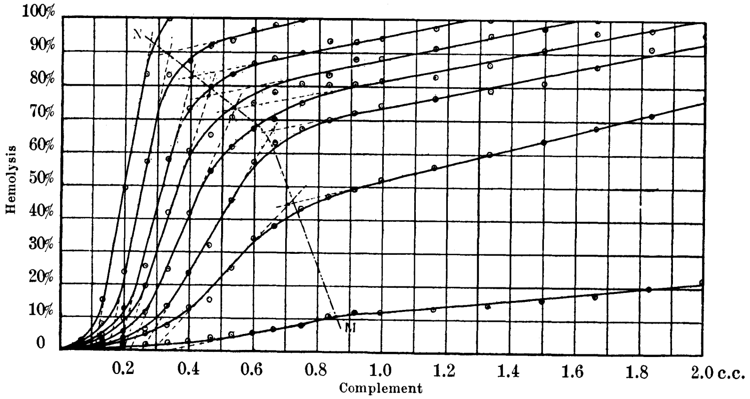


FIG. 6.—Parallel complement curves. (Immune Serum No. 2, Normal Serum No. 4.) The curves read from below upwards were made with the following amounts of amboceptor: .01 c.c., .02 c.c., .03 c.c., .04 c.c., .05 c.c., .06 c.c., .08 c.c., .12 c.c.

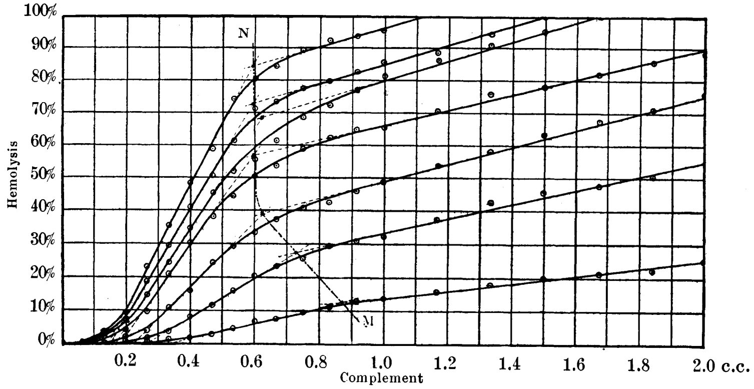


FIG. 7.—Parallel complement curves. (Immune Serum No. 1, Normal Serum No. 5.) The curves read from below upwards were made with the following amounts of amboceptor: .03 c.c,, 0.6 c.c., .09 c.c., .12 c.c., .15 c.c., .18 c.c., .21 c.c.

From the curves thus far recorded, it is seen that the typical complement curve (Fig. 8) can be divided into three parts: an initial part AB, characterized by a slow, gradually increasing rise; an intermediate part BC, characterized by a rapid, apparently uniform rise; and a third part CD, characterized by a slow, apparently uniform rise.

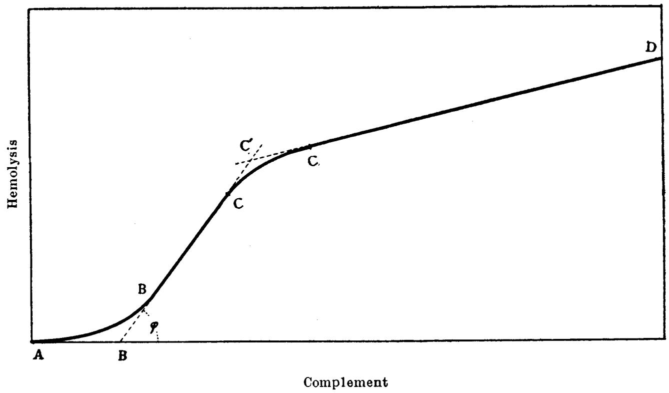


FIG. 8.—The typical complement curve.

An examination of the curves in Figs. 6 and 7 shows that the length of the portion AB decreases with increase in the amount of amboceptor used, the relation between the two being expressed, at least approximately (using B′, the point most accurately determined experimentally) by the equation:

$$AB' = \frac{K^{*}}{\sqrt{\text{Amboceptor}}} \qquad \text{(i)}$$

or,

$$AB' \times \sqrt{\text{Amboceptor}} = K \qquad \text{(ii)}$$

The values of K, calculated for the curves in Figs. 6 and 7, are given below, and show a striking uniformity in each series.

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* A constant.

AB	Amboceptor	$\sqrt{\text{Amboceptor}}$	$K = AB \times \sqrt{\text{Amboceptor}}$
17.0	.01	.100	1.7
14.5	.02	.141	2.0
13.0	.03	.173	2.3
11.0	.04	.200	2.2
10.0	.05	.224	2.2
9.2	.06	.245	2.3
7.8	.08	.283	2.2
5.6	.12	.346	1.9
			Average, 2.1
17.5	.03	.173	3.0
13.2	.06	.245	3.2
11.0	.09	.300	3.3
9.0	.12	.346	3.1
8.5	.15	.387	3.2
7.5	.18	.424	3.2
7.0	.21	.458	3.2
			Average, 3.2

The rate of rise of the part BC increases with increase in the amount of amboceptor present. The relation between the two is expressed, at least approximately, by the equation:

$$\tan \phi = K \times \text{Amboceptor} \tag{iii}$$

or,

$$\frac{\tan \phi}{\text{Amboceptor}} = K \tag{iv}$$

The values of K, calculated for the curves in Figs. 6 and 7 are given below and are fairly constant in each series.

ϕ	$\tan \phi$	Amboceptor	$K = \frac{\tan \phi}{\text{Amboceptor}}$
12°	0.21	.01	(21)
48	1.1	.02	55
60	1.7	.03	57
66	2.2	.04	55
71	3.0	.05	60
73	3.3	.06	55
76	4.0	.08	50
80	5.7	.12	48
			Average, 54

ϕ	tan ϕ	Amboceptor	$K = \frac{\tan \phi}{\text{Amboceptor}}$
14°	0.25	.03	**8**
31	0.6	.06	**10**
44	0.97	.09	**11**
55	1.4	.12	**12**
57	1.5	.15	**10**
60	1.7	.18	**9.5**
61	1.8	.21	**9**
		Average,	**10**

The height to which C rises increases with increase in the amount of amboceptor present. That this increase follows definite laws is shown in Figs. 6 and 7 by the curve MN, the locus of C′. The loci differ in the two series, indicating that the law governing C′ is either a quite complex one or that there are qualitative or quantitative differences between the sera used.

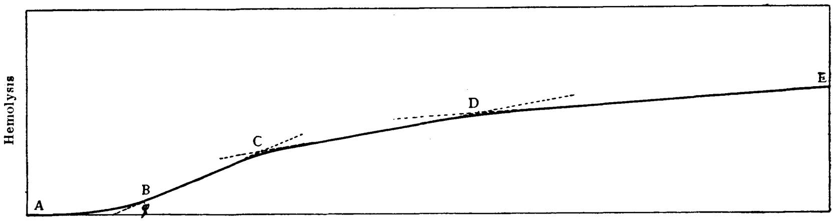

FIG. 9.—Complement curve with minimal amount of amboceptor.

The rate of rise of the part CD is, except when very small amounts of amboceptor are used, practically independent of the amount of amboceptor present.

In order to determine whether CD continues indefinitely as a straight line or not, curves were run with very small amounts of amboceptor and correspondingly large amounts of complement. Two such curves are shown in the upper half of Fig. 15. The upper curve was made with 0.018 c.c. of heated immune serum (No. 2) as amboceptor, the lower curve with 0.0135 c.c. of the same serum. The complement was increased to five c.c. in each curve.

From these curves it is seen that to the complement curves previously obtained there must be added a fourth part, DE (Fig. 9), also apparently a straight line. The rate of rise of DE is, at least approximately, independent of the amount of amboceptor present. Whether DE continues indefinitely as a straight line or not has as yet not been determined.

Attempts to express the complete complement curve as a mathematical equation have thus far been unsuccessful.

THE AMBOCEPTOR.

In a similar way curves were plotted showing the change in hemolytic power as the amboceptor varies. To do this, increasing amounts of amboceptor were added to a series of tubes, the volumes made up to a constant quantity, a constant amount of complement added, and corpuscles exposed to the action of the mixtures. A number of curves obtained in this way are shown in Fig. 10.

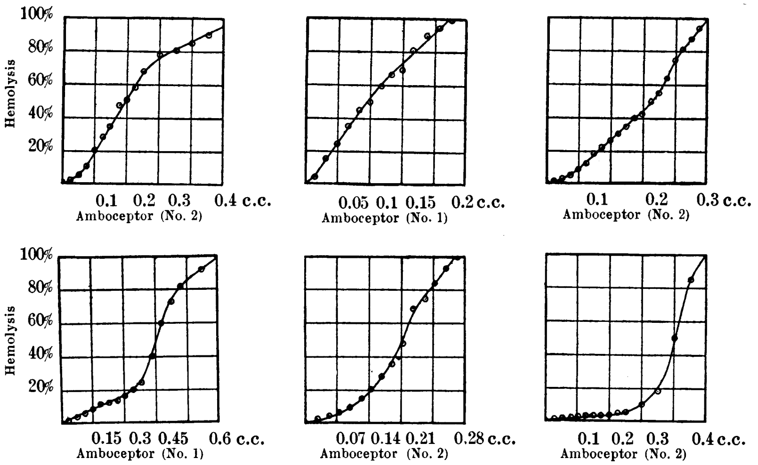

FIG. 10.—Amboceptor curves. Read from above the curves were made with the following amounts of complement: 0.33⅓ c.c., 0.46⅔ c.c., 0.20 c.c., 0.26⅔ c.c., 0.23⅓ c.c., 0.06⅔ c.c.

To determine the effects of the amount or complement on the nature of these curves, parallel curves were run as before, with the same increasing amounts of amboceptor, but with different constant amounts of complement. A series of such curves is shown in Fig. 11.

Three additional series of parallel amboceptor curves, are shown in Figs. 12, 13 and 14. In Fig. 14 quite small amounts of com-

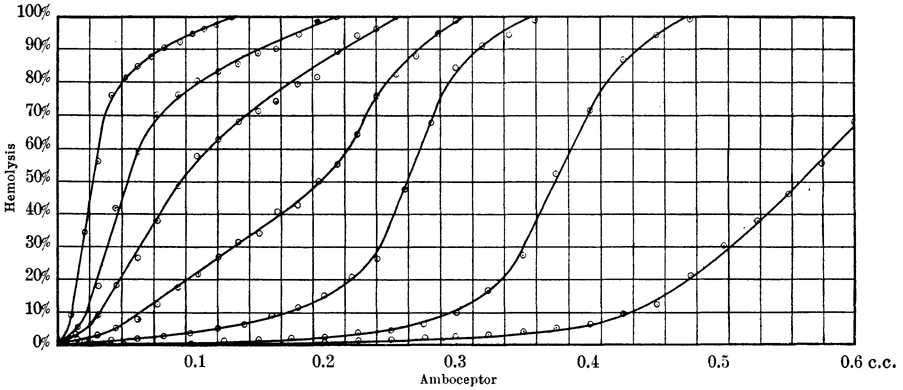

FIG. 11.—Parallel amboceptor curves. (Normal Serum No. 3, Immune Serum No. 2.) Read from below upwards, the curves were made with the following amounts of complement: 0.03⅓ c.c., 0.06⅔ c.c., 0.13⅓ c.c., 0.20 c.c., 0.26⅔ c.c., 0.33⅓ c.c., 0.53⅓ c.c.

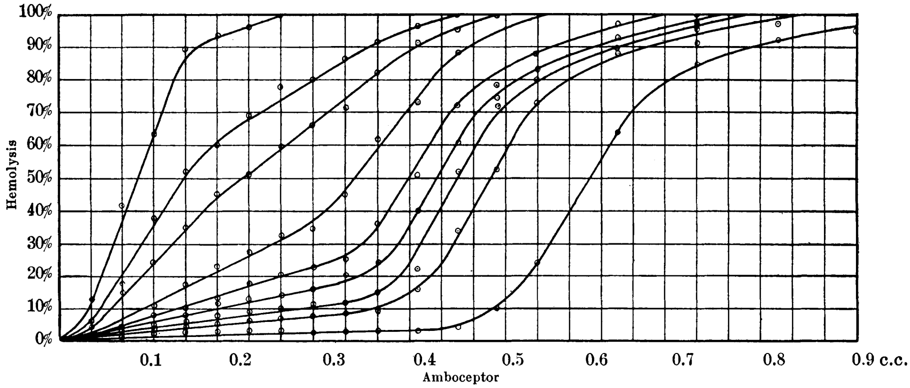

FIG. 12.—Parallel amboceptor curves. (Normal Serum No. 3, Immune Serum No. 1.) Read from below upwards, the curves were made with the following amounts of complement: 0.13⅓ c.c., 0.2 c.c., 0.23⅓ c.c., 0.26⅔ c.c., 0.3 c.c., 0.36⅔ c.c., 4.6⅔ c.c., 0.56⅔ c.c., 0.9 c.c.

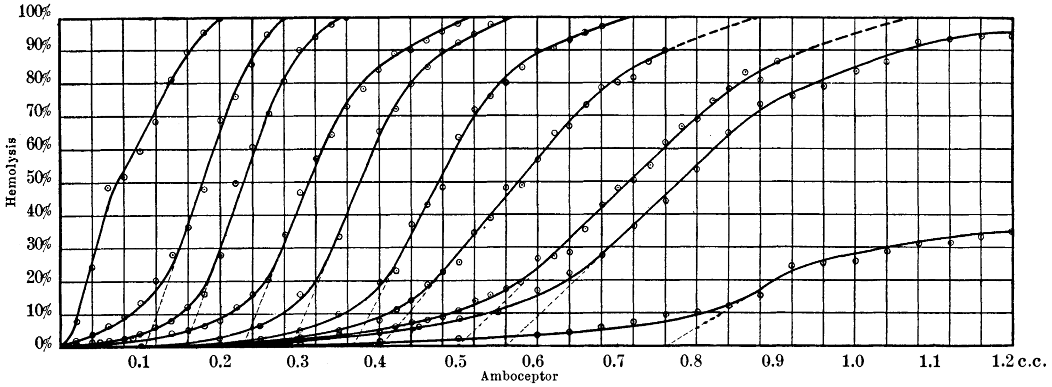

FIG. 13.—Parallel amboceptor curves. (Normal Serum No. 4, Immune Serum No. 2.) Read from below upwards, the curves were made with the following amounts of complement: 0.015 c.c., 0.0225 c.c., 0.03 c.c. 0.04 c.c., 0.05 c.c., 0.06⅔ c.c., 0.10 c.c., 0.16⅔ c.c., 0.23⅓ c.c., 0.36⅔ c.c.

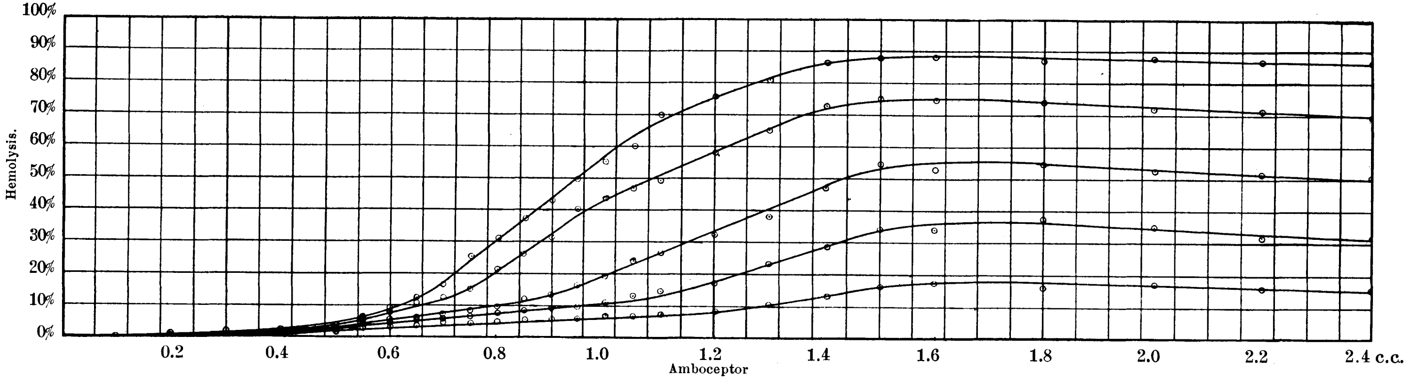

FIG. 14.—Amboceptor curves with minimal amounts of complement. (Normal Serum No. 3, Immune Serum No. 2.) Read from below upwards, the curves were made with the following amounts of complement: 0.01 c.c., 0.0125 c.c., 0.015 c.c., 0.0175 c.c., 0.02 c.c., 0.0225 c.c.

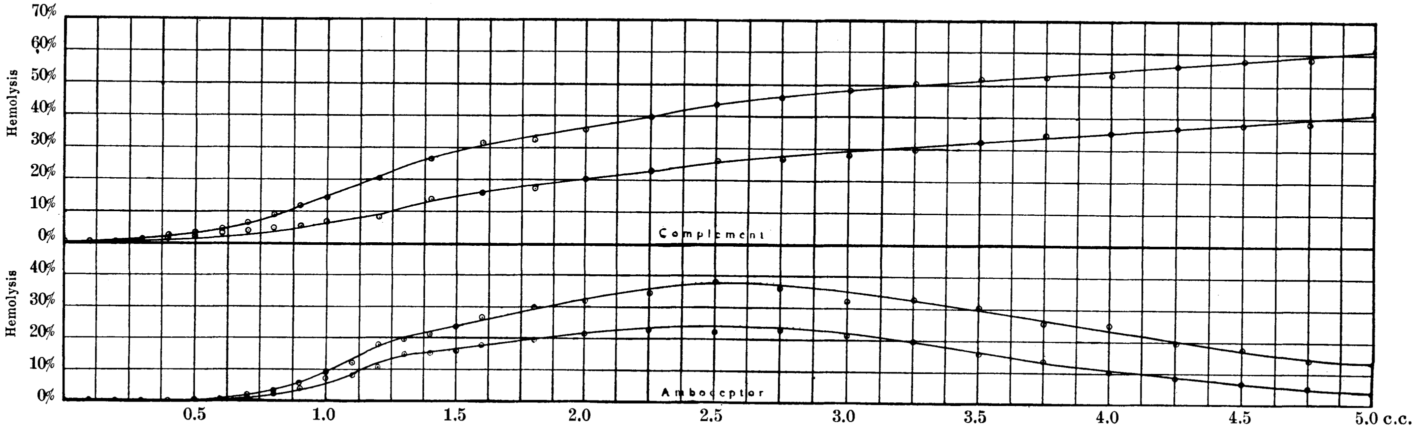

FIG. 15.—Complement and amboceptor curves compared. (Normal Serum No. 4, Immune Serum No. 2.) The two lower curves were made with 0.0135 c.c. and 0.018 c.c. of complement, the two upper with the same amounts of amboceptor. The curves were made on the same day and with the same corpuscles.

plement were used, and correspondingly large amounts of amboceptor. A second set of curves made with very small amounts of complement and still larger amounts of amboceptor is shown in the lower half of Fig. 15.

From these curves it is seen that the typical amboceptor curve (Fig. 16) consists of four parts: an initial part AB, characterized by a slow, either uniform or gradually increasing rise (AB apparently differing with the two immune sera used); a second part BC, characterized by a rapid, uniform rise; a third part, CD characterized by a slow, uniform rise; and a fourth part, DE, characterized by a slow, gradually decreasing fall.

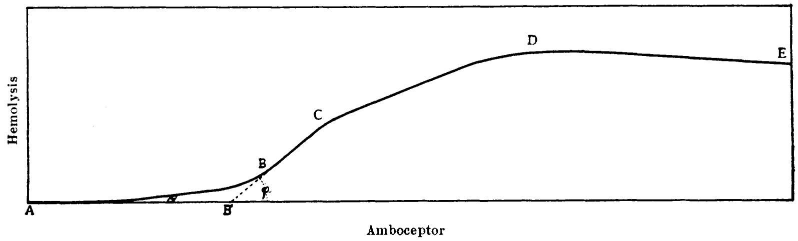

FIG. 16.—The typical amboceptor curve.

The length of the portion AB decreases with increase in the amount of complement used. The quantitative relation between the two is shown in the following table, taken from Fig. 13, from which it is seen that

$$AB \times \sqrt{\text{Complement}} \text{ not equal to K, a constant.}$$

a relation that might have been expected from a study of complement curves.

Complement	A B′	$\sqrt{\text{Complement}}$	A B′ × $\sqrt{\text{Complement}}$
0.01½	19.1	.123	**2.35**
0.02¼	14.0	.146	**2.04**
0.03	12.5	.173	**2.16**
0.04	10.1	.200	**2.02**
0.05	9.2	.224	**2.06**
0.06⅔	7.2	.258	**1.86**
0 10	5.7	.317	**1.80**
0.16⅔	3.9	.411	**1.60**
0.23⅔	2.7	.472	**1.27**

The rate of rise of that part of AB that is represented by a straight line, increases with increase in the amount of complement present. The relation between the two is shown in the following table, taken from Fig. 12, from which it is evident that

$$\frac{\tan \theta}{\text{Complement}} \text{ not equal to K}$$

a relation that might have been expected from a study of complement curves.

Complement	θ	$\tan \theta$	$\frac{\tan \theta}{\text{Complement}}$
0.1⅓	2°	0.03	**0.2**
0.2	4°	0.09	**0.5**
0.2⅓	7° 30′	0.13	**0.6**
0.2⅔	11°	0.20	**0.8**
0.3	17°	0.30	**1.0**
0.3⅔	24°	0.44	**1.2**
0.4⅔	42°	0.90	**1.9**
0.5⅔	52° 30	1.30	**2.3**
0.9	69°	2.60	**2.9**

The rate of rise of the part BC increases, when small quantities of complement are used (Figs. 13 and 14), with increase in complement. With larger amounts of complement (Figs. 12 and 13) the rate is practically independent of the amount of complement present. The relation between the two is shown in the following table, taken from Fig. 13, from which it is evident that

$$\frac{\tan \phi}{\text{Complement}} \text{ not equal to K}$$

a relation that might have been expected from a study of complement curves.

Complement	ϕ	$\tan \phi$	$\frac{\tan \phi}{\text{Complement}}$
0.01½	29°	0.55	**37**
0.02¼	43°	0.92	**41**
0.03	44°	0.96	**32**
0.04	48° 30′	1.12	**28**
0.05	61°	1.80	**36**
0.06⅔	66°	2.27	**34**
0.10	67°	2.33	**23**
0.16⅔	69° 30′	2.67	**16**
0.23⅔	72°	3.00	**13**
0.36⅔	74° 30′	3.60	**10**

The height to which BC rises increases, when small amounts of complement are used, with increase in complement. With larger amounts of complement it is practically constant. No mathematical expression has yet been found for this height. The length BC decreases when large amounts of complement are used, due to the increase in the height of B, until, with very large amounts, BC = O.

The rate of rise of the part CD (Figs. 14 and 15) is practically independent of the amount of complement present. The height to which D rises (maximum hemolysis) bears no simple relation to the amount of complement present, the relation between the two being shown in the following table:

Complement	Maximum Hemolysis
.01. c.c.	18%
.01¼	27
.01½	55
.01¾	76
.02	88
.02¼	92

The rate of fall of DE (Figs. 14 and 15) is very nearly independent of the amount of complement present.

The difference between the amboceptor and complement curves is plainly shown in Fig. 15, the curves of which were made with the same sera, the same corpuscles, and on the same day. In this series the routine technique was varied by making up the volumes in the various tubes with $\frac{m}{8}$ NaCl in place of the dilute heated normal serum heretofore used. This change, apparently, made no difference in the general nature of the curves.

Attempts to express the complete amboceptor curve as a mathematical equation have thus far been unsuccessful.

THE WHOLE SERUM.

Experiments were also undertaken to determine the effect on hemolytic power of varying both complement and amboceptor simultaneously. To do this, increasing amounts of immune serum or of an immune serum mixture were placed in a series of hemolytic tubes, the volumes made up to a constant quantity, corpuscles added, and curves plotted as before. A curve obtained in this way is shown in Fig. 17.

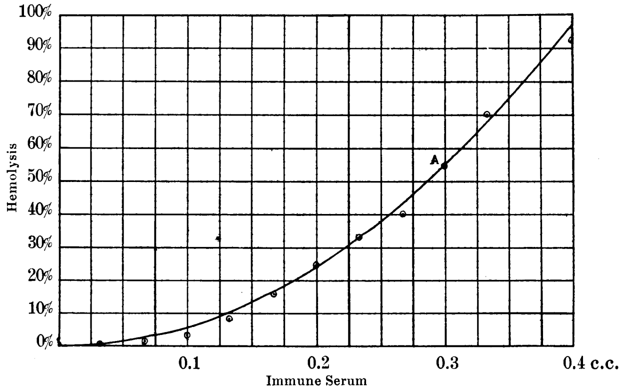

FIG. 17.—The curve of squares. (Immune Serum No. 1.)

This curve was apparently so nearly the parabola determined by the equation

$$\text{Hemolysis} = K\,(\text{Serum})^2$$

that in drawing it an accurately determined point, A, was selected as a point of true observation and the curve calculated mathematically from it. The agreement between the observed hemolysis and the hemolysis calculated from this assumption is shown below:

Serum	Hemolysis (obs.)	Hemolysis (calc.)
c.c.	%	%
0.00	0	0
0.03⅓	0+	1.5
0.06⅔	1.5	3
0.10	4−	6
0.13⅓	8.5	11
0.16⅔	16	17
0.20	25	24
0.23⅓	33	33
0.26⅔	40	43
0.30	55	[55]
0.33⅓	70	68
0.40	92.5	97

A second curve obtained with the same serum two weeks later is shown in Fig. 18. The relation between the observed and calculated hemolysis in this curve is shown below:

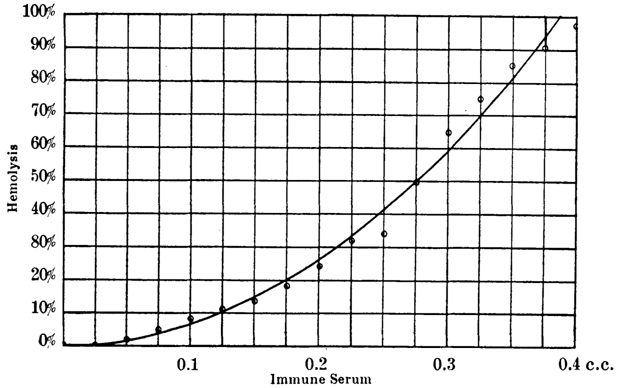

FIG. 18.—The curve of squares. (Immune Serum No. 1.)

Serum c.c.	Hemolysis (obs.) %	Hemolysis (calc.) %
0.00	0	0
0.025	0+	0.4
0.05	2.5	1.6
0.075	5	3.8
0.10	8.5	6.6
0.125	11.25	10.3
0.15	13.75	15
0.175	18.5	20
0.20	24	26.5
0.225	33	33
0.25	34	41
0.275	50	[50]
0.30	65	60
0.325	75	70
0.35	85	81
0.375	90	93
0.40	95	106

From this agreement a law might readily have been deduced that, the volume and amount of corpuscles remaining constant, the amount of hemolysis increases as the square of the amount of serum used, were it not for the fact that the serum from the second immune goat gave a totally different curve (Fig. 19).

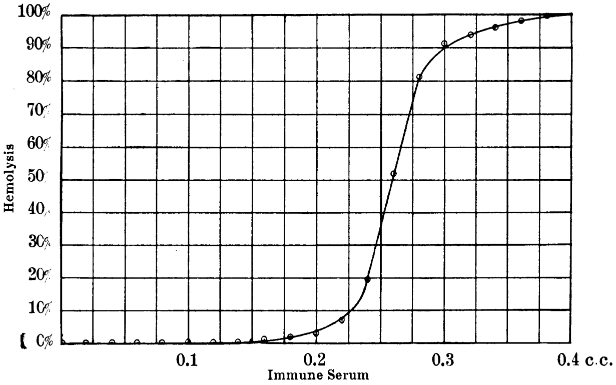

FIG. 19.—Whole-serum curve. (Immume Serum No. 2.)

Three months later, after eight additional injections, a curve similar to this was obtained from the goat that had formerly given the simpler result (Fig. 20), at which time the second immune goat gave an even more striking dissimilar curve (Fig. 21).

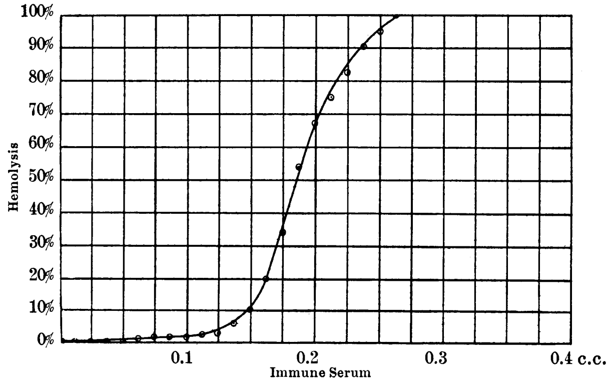

FIG. 20.—Whole-serum curve. (Immune Serum No. 1, three months later.)

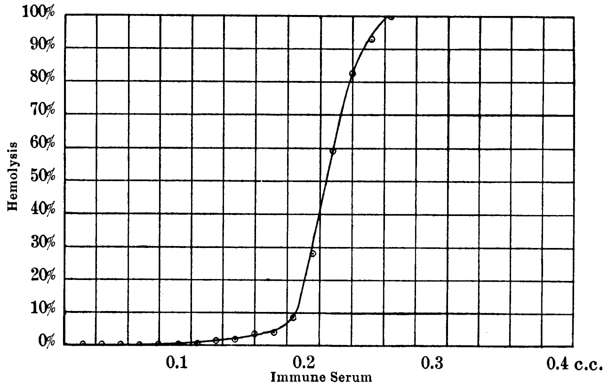

FIG. 21.—Whole-serum curve. (Immune Serum No. 2, three months later.)

It was suspected from this change that the apparent parabolas obtained above were but special cases of more complex curves. A re-examination of data showed this in reality to be the case, the true hemolytic curve being, even in these instances, a curve of double curvature. The relation between this true curve and the calculated parabola is shown in Fig. 22, which was made from the data of Fig. 18.

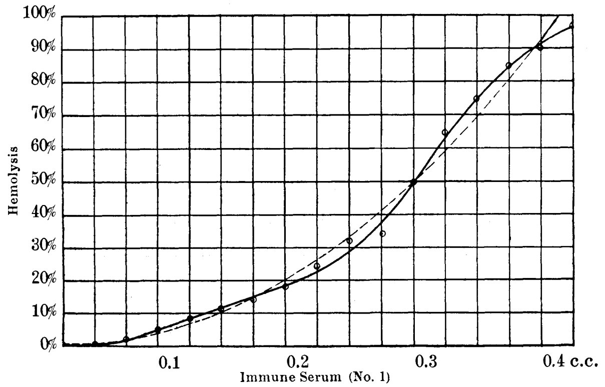

FIG. 22.—Whole-serum curve and curve of squares compared.

As the curve changed with repeated injections, it was thought that its nature might depend on the relative amount of complement and amboceptor present. To test this hypothesis, curves were plotted with various mixtures of normal serum (complement) and heated immune serum (amboceptor). A number of these are shown in Fig. 23.

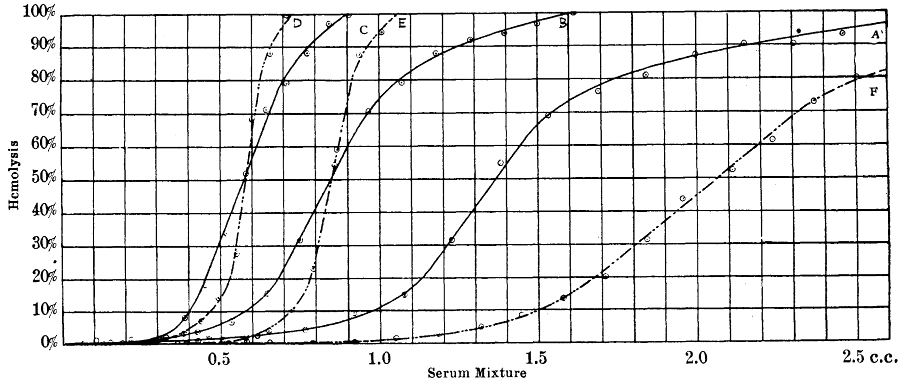

FIG. 23.—Whole-serum curves. (Immune Serum No. 1, Normal Serum No. 4.) Read in alphabetic order the curves were made with the following approximate amboceptor-complement ratios: 1:40, 1:13, 1:3, 1:1, 5:1, 20:1.

By interpolating a curve between A and B a curve is obtained that approximates a parabola. This curve would correspond to an approximate amboceptor-complement ratio 1 : 20 (each measured in the arbitrary units). A curve interpolated between B and C would approximate Fig. 19, and would correspond roughly to an amboceptor-complement ratio 1 : 8. A curve between D and E would correspond roughly to Fig. 20, and to an approximate ratio 3 : 1.

Attempt was made to reproduce the parabola with an artificial amboceptor-complement mixture. This was successful (Fig. 24). The agreement between the calculated and observed hemolysis in this curve is shown below:

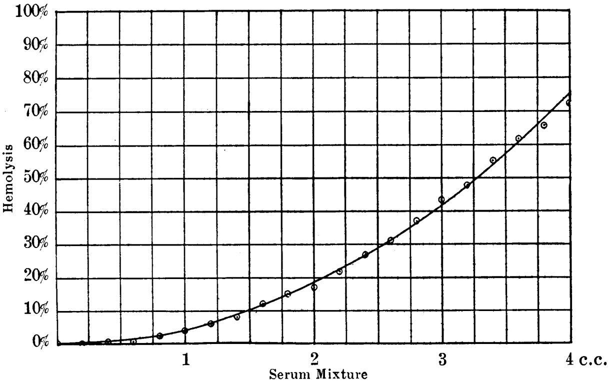

FIG. 24.—Coincident whole-serum curve and curve of squares. The curve was made with a mixture of heated immune serum No. 2 and normal serum No. 3, in the amboceptor-complement ratio 1:200.

Serum c.c.	Hemolysis (obs.) %	Hemolysis (calc.) %
0.0	0	0
0.2	0	0.2
0.4	0.25	0.7
0.6	0.5	1.7
0.8	2.5	3
1.0	4	4.7
1.2	6	6.7
1.4	8	9.2
1.6	12	12
1.8	15	15.2
2.0	17	18.8
2.2	22	22.7

Serum c.c.	Hemolysis (obs.) %	Hemolysis (calc.) %
2.4	27	27
2.6	31	31.7
2.8	37	36.7
3.0	43	42.2
3.2	48	[48]
3.4	55	53.6
3.6	62	60.8
3.8	66	67.7
4.0	72	75

The typical whole-serum curve (Fig. 25) can be divided into four parts: an initial part, AB, characterized by no hemolysis; a second part, BC, characterized by a gradually increasing rise; a third part, CD, characterized by a rapid, uniform rise; and a fourth part, DE, characterized by a slow, gradually decreasing rise.

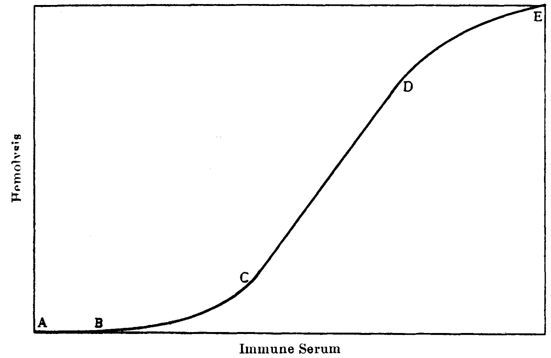

FIG. 25.—The typical whole-serum curve.

The length AB is roughly proportional to the amount of amboceptor present, the relation between the two being shown by the following table, taken from Fig. 23:

Amboceptor	AB
0.075 c.c.	1—
0.15	1.5—
0.30	2—
0.60	2.5
1.20	4.5
2.50	9

The rate of rise of the part CD is greatest when the amboceptor and complement are approximately equal in amount, and decreases as either predominates. The height to which CD rises is, in the curves thus far obtained, apparently independent of the relative amounts of complement and amboceptor used. No simple mathematical relations have been discovered.

Attempts to express the whole-serum curve as a mathematical equation have thus far been unsuccessful.

THE CORPUSCLES.

Experiments were also undertaken to determine the effect of changes in the number of corpuscles on the amount of hemolysis produced. To determine this, increasing amounts of corpuscles were added to equal amounts of immune serum or to an immune serum mixture, and the hemolysis estimated colorometrically, as percentages of total hemolysis when 0.25 c.c. of corpuscles are used.

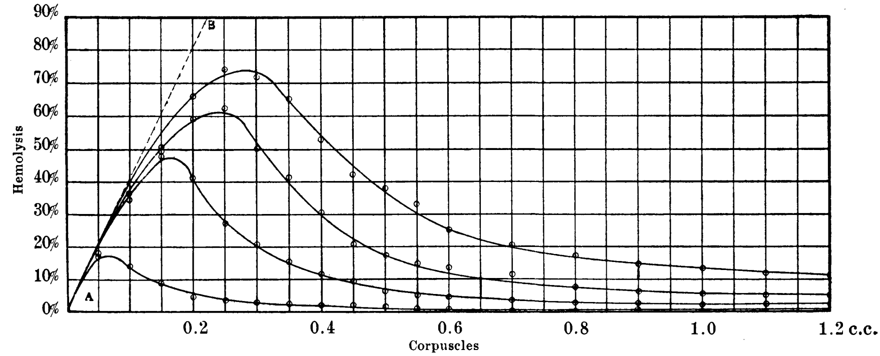

FIG. 26.—The corpuscle curve. Read from below upwards the curves were made with the following amounts of immune serum No. 2: 0.13⅓ c.c., 0.16⅔ c.c., 0.20 c.c., 0.23⅓ c.c.

A number of curves obtained in this way are shown in Fig. 26. From these it is seen that as the amount of corpuscles increase, the amount of hemoglobin liberated increases, at first rapidly, but soon falls short of the tint that would have been obtained were all the corpuscles present dissolved. The curve of complete lysis is represented by AB. The amount of hemoglobin liberated soon reaches a maximum, and then rapidly decreases, presumably eventually to zero.

With the larger quantities of serum used, the maximum tint was roughly proportional to the amount of serum present, a relation, however, that did not hold when smaller amounts of serum were present. The available data is too meager to determine the exact ralation between the two. The relation in Fig. 26 is shown below:

Serum	Maximum Hemolysis
0.13⅓ c.c.	18%
0.16⅔	48
0.20	62
0.23⅓	74

No mathematical expression for the corpuscle curve as a whole has as yet been obtained.

CONCLUSION.

The curves here reported are part of work preliminary to the study of neutralization curves for hemolytic sera. They furnish, however, data by means of which some of the physico-chemical laws proposed by Arrhenius can be tested. A mathematical analysis of these data with this object in view will be the subject of a separate report.